

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/30, 47/20	A1	(11) International Publication Number: WO 97/14430 (43) International Publication Date: 24 April 1997 (24.04.97)
(21) International Application Number: PCT/SE96/01302 (22) International Filing Date: 14 October 1996 (14.10.96) (30) Priority Data: 9503679-4 20 October 1995 (20.10.95) SE (71) Applicant (for all designated States except US): PHARMACIA & UPJOHN AB [SE/SE]; S-112 87 Stockholm (SE). (72) Inventors; and (75) Inventors/Applicants (for US only): FRANSSON, Jonas [SE/SE]; Torkelsgatan 14 B, S-753 29 Uppsala (SE). LUNDIN, Ronny [SE/SE]; Lobovägen 3, S-178 32 Ekerö (SE). (74) Agents: TANNERFELDT, Agneta et al.; Pharmacia & Upjohn AB, S-112 87 Stockholm (SE).		(81) Designated States: AU, CA, FI, JP, NO, NZ, RU, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: USE OF THIOETHERS AS ANTIOXIDANT FOR PEPTIDES AND PROTEINS AND COMPOSITIONS CONTAINING THE THIOETHERS (57) Abstract The claimed invention relates to the use of special thioethers as antioxidant for peptides and proteins, especially IGF-I. It also relates to a method for inhibiting the oxidation of peptide or protein in a solution containing the peptide or protein, characterized by the addition of the compounds and to compositions comprising the peptide or protein and the compound.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

USE OF THIOETHERS AS ANTIOXIDANT FOR PEPTIDES AND
5 PROTEINS AND COMPOSITIONS CONTAINING THE THIOETHERS.

The claimed invention relates to the use of special thioethers as
10 antioxidant for peptides and proteins, especially IGF-I .

It also relates to a method for inhibiting the oxidation of peptide or protein
in a solution containing the peptide or protein, characterized by the
addition of the compounds and to compositions comprising the peptide or
protein and the compound.

15
Introduction

The stability of proteins is generally a problem in the pharmaceutical industry.
The stability of proteins and peptides is crucial during both processing and
20 storage.

A formulation with a low amount of protein will generally lose activity during
purification, sterile manufacturing, storage and during the administration.
It has often been solved by drying of the protein in different drying processes,
such as freeze-drying. The protein has thereafter been distributed and stored in
25 dried form. The patient necessarily has to reconstitute the dried protein in a
solvent before use, which of course is a disadvantage and is an inconvenience
for the patient.

It would thus facilitate the use of a pharmaceutical protein if it can be produced
30 and distributed as a stable solution with a prolonged storage life to the patient,
who could inject the medicament directly without reconstitution.

It would be advantageous if the final pharmaceutical solution only contains a minimum of additives.

Proteins are different with regard to physiological properties. When preparing a pharmaceutical preparation which should be physiologically acceptable and stable for a long time, consideration can not only be taken to the physiological properties of the protein but also other aspects must be considered such as the industrial manufacture, easy handling for the patient and safety for the patient.

Insulin-like Growth Factor I (IGF-I) is a peptide present in plasma and other body fluids as well as many cells/tissues. It comprises 70 amino acids, including 3 disulphide bonds, and can stimulate proliferation of a wide range of cell types and it mediates some of the effects of growth hormone. Human IGF-I has been purified from plasma and its complete amino acid sequence is established. (Rinderknecht E et al. "The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin" J. Biol. Chem 253; 2769-76, 1978). Sequences with extensive homologies to human IGF-I are present in IGF-I purified from plasma of other species.

Because of the scarcity of purified plasma IGF-I there was a great necessity to develop methodology for the commercial scale production of IGF-I. Nowadays, such large scale production can readily be achieved by using recombinant DNA techniques.

As a result of studies with preparations of recombinant DNA IGF-I it has been demonstrated that it promotes skeletal growth and skeletal muscle protein synthesis. IGF-I has been shown to act both as an endocrine factor as well as a paracrine/autocrine factor. (Skottner et al, Endocrinology, Vol. 124, No 5, 1989 and Cook et al, J Clin Invest 81; 206-212; 1988)

Moreover, IGF-I is also effective for the treatment or prevention of catabolic states in patients (Swedish patent application SE 9002731-9) and improves the regeneration of transected peripheral nerves (EP 0 308 386).

It has previously been demonstrated *in vitro* that IGF-I also can promote actin synthesis in myocytes in culture (Florini, J R, Muscle and Nerve 10 (1987) 577-598 and contractility of neonatal rat cardiocytes *in vitro* (Vetter, U *et al.*, Basic Res. Cardiol. 83 (1988)647-654).

5 Other pharmaceutical uses of IGF-I have also been suggested.

In WO 92/15614, Chiron, methionine is used for stabilisation of growth factors.

One disadvantage with methionine as antioxidant for therapeutical
10 formulations, is the fact that it has animal origin. This should preferably be avoided.

The problem to solve was thus to find an antioxidating agent which was at least as good as methionine but without the disadvantage being of animal origin.

15 Although methionine is a known as antioxidant, no other compounds which are chemically similar to methionine has earlier been suggested as an antioxidant.

It has now been found that special antioxidants can give a peptide
20 solution, especially a growth factor solution, stable up to 18 months.

The stability for a IGF-I solution has been compared to solutions without antioxidants and to a solution containing methionine as antioxidants.

It must be regarded as surprising that the compounds according to
25 formula I in claim 1 have at least the same stability as the earlier known methionine but being chemically totally pure.

This is a novel and surprising finding.

The present invention thus relates to the use of



in which

- 5 R_1 is a carbon chain, preferably with 1-8 carbon atoms and more preferably with 1-4 carbon atoms,
 R_2 is alkyl, preferably with 1-8 carbon atoms and more preferably methyl, ethyl, propyl or butyl
 X is a rest of an amino acid, amino alcohol or hydroxy group
10 such as $\text{NH}_2\text{-CH-COOH}$, $\text{NH}_2\text{-CH-(CH}_2\text{OH)}$ or OH
or X , R_1 and/or R_2 form a ring structure,
e.g. morpholine ring,
with the proviso that (I) cannot be methionine,
as antioxidants for peptides and proteins.
15 X should preferably not be an acid such as $\text{-CH}_2\text{-COOH}$ or CH(OH)-COOH when stabilising some sensitive proteins, e.g. IGF-I.
Ethionine, Butionine, 2-(methylthio)-ethanol, S-methyl-L cystein and L-methininol are preferred as antioxidazing agent especially for IGF-I.
20 The invention also relates to a method for inhibiting the oxidation of peptide or protein in a solution containing the peptide or protein and for inhibiting unwanted oxidation of methionine, tryptophane, cysteine and/or histidine residues during processing steps such as refolding or chromatographic procedures, characterized by the addition of a
25 compound according to formula I.

The peptide is preferably a growth factor and more preferably IGF-I.
As growth factor could be mentioned Epidermal Growth factor (EGF)
Nerve Growth Factor (NGF), Platelet Derived Growth Factor (PDGF) etc.

The added substances prevent the rapid oxidation of the amino acids methionine, tryptophane, cysteine and histidin and thereby increases the shelf-life considerably of a pharmaceutical peptide and protein preparations containing these amino acids.

5

Composition comprising peptides and proteins, preferably growth factors and more preferably IGF-I, and a compound according to formula (I) as antioxidant are also claimed.

10

EXAMPLE

The recombinant human IGF-I (rhIGF-I) used in the experiments was produced in yeast. rhIGF-I was initially synthesised as a hybrid protein fused to the yeast α -mating factor pre-pro leader peptide. After expression the primary translation product was secreted out of the cell. During this process the pre-pro-leader was cleaved off. Correctly processed and secreted rhIGF-I could then be isolated from the fermentation media in its native form.

20

The media with rhIGF-I was then micro filtered and impurities were removed by several chromatographic techniques known within the field.

In the example solutions of IGF-I pools from the final step in the purification process were ultrafiltered to obtain a correct concentration and the correct buffer formulation.

25

The samples were stored at $+7\pm 1^\circ\text{C}$ and $25\pm 1^\circ\text{C}$.

30 The following analytical techniques were used in all examples:

Reversed Phase HPLC (RP-HPLC) The elution system is composed of acetonitrile, water, phosphate buffer and propane sulphonic acid sodium salt. Elution is accomplished by decreasing the polarity of the mobile phase. UV detection at 220 nm. Used for measurement of oxidised IGF-I.

- 5 The remains of the original concentration is calculated in per cent.

All chemicals (except IGF-I) were of p.a. grade or better and were purchased from regular manufacturer and used as received.

- 10 IGF-I in a phosphate buffer solution was mixed with different antioxidants to a final composition per mL of:

	IGF-I	0,13 mmol (1 mg)
	Phosphate buffer	10 mmol
15	Sodium Chloride	145 mmol
	antioxidant	6,5 mmol
	distilled water	to make 1,0 mL
	pH	5,9

- 20 The antioxidants were added in excess in a molar relation to IGF-I of 50:1. The solutions were sterile filtered and dispensed in glass vials in portions of 1 mL. The samples were stored at +7°C and +25°C, protected from light. After storage the samples were analysed with reversed phase HPLC for oxidised methionine in IGF-I.

- 25 Table I shows the amounts of oxidised protein after storage.

Table I

% oxidised IGF-I

5	Agent	at start	2 weeks +25°C	1 month +25°C	6 months +7°C	18 months +7°C
<hr/>						
10	Reference with no added antioxidant present	1.4	ND	ND	ND	2.4
	Methionine	1.3	1.3	1.5	1.5	ND
	<u>Agents according to the invention:</u>					
15	Ethionine	1.4	ND	1.5	ND	1.5
	Methioninol	1.4	1.5	ND	ND	ND
	2-(methylthio) ethanol	1.3	1.3	ND	1.5	ND
	S-methyl-L-cysteine	1.8	1.7	ND	2.1	ND
	Buthionine	1.3	1.3	ND	1.4	ND
20	Methylthio acetic acid	1.4	ND	1.5	ND	ND
	alfa-keto-gamma- methylthiobutyric acid	1.4	ND	1.5	ND	ND
	<u>Agents for comparison:</u>					
25	Ascorbic acid	1.0	5	ND	ND	ND
	disodium EDTA	1.4	ND	ND	2.2	2.3
	N-Acetylcystein	ND	*	*	*	*
	Sodium thiosulphate	1.2	ND	11.9	ND	ND
	1-4-dithiothreitol(DTT)	ND	*	*	*	*
30	n-Propylgallate	ND	*	*	*	*
	Glutathione (reduced)	ND	*	*	*	*
	Sodium bisulphite	1.7	*	*	*	*
	Thiomorpholine HCL	1.9	2.8	ND	ND	ND
	L-thiazolidine-4-					
35	carboxylic acid	1.9	3.6	ND	ND	ND
	L-Tryptophane	ND	*	*	*	*
	Homocysteine	1.0	*	*	*	*
	Homocystine	1.0	*	*	*	*

40 *) Precipitated protein was found.

ND=not determined

A. The following substances have been investigated and compared to other compounds and found to function as an antioxidation agent for IGF-I:

- 5 Methionine
- Ethionine
- Buthionine
- 2-(methylthio)-ethanol
- S-Methyl-L-cystein
- 10 Methioninol

B. The following substances have been investigated and can function as antioxidation agent but decrease the stability for IGF-I and are not

- 15 suitable for that protein:
- (Methylthio) acetic acid
- alpha-keto-gamma-methiolbutyric acid.

Common antioxidants showed no protective effect on the oxidation of IGF-I. Chelating compounds such as sodium EDTA had no effect on the oxidation rates while ascorbic acid and sodium thiosulphate even increased the oxidation. Many of the common antioxidants such as glutathione or sodium bisulphite and many of the new substances precipitated the protein.

25 The substances that prevented oxidation and did not precipitate the protein all contained a sulphur surrounded by two carbons. When -SH groups were available the protein was precipitated as they reacted with disulphide bridges.

CONCLUSION

Only substances that contained a sulphur surrounded by two carbon
5 groups prevented oxidation. Conventional antioxidants were unable to
prevent oxidation and some even enhanced the oxidation.

CLAIMS

5

1. Use of



in which

10 R₁ is a carbon chain, preferably with 1-8 carbon atoms and more preferably with 1-4 carbon atoms,

R₂ is alkyl, preferably with 1-8 carbon atoms and more preferably methyl, ethyl, propyl or butyl

X is a rest of an amino acid, amino alcohol or hydroxy group or X, R₁

15 and/or R₂ form a ring structure,
with the proviso that (I) cannot be methionine,
as antioxidants for peptides and proteins.

2. Use according to claim 1 in which the peptide is a growth factor and

20 preferably Insulin-like growth factor I (IGF-I) .

3. Use according to claim 1 or 2 in which the antioxidant is chosen in the group consisting of Ethionine, Butionine, 2-(methylthio)-ethanol, S-methyl-L cystein and L-methininol.

25

4. Method for inhibiting the oxidation of peptide or protein in a solution containing the peptide or protein, characterized by the addition of a compound according to formula I.

- 5 5. Method for inhibiting unwanted oxidation of methionine, tryptophane, cysteine and/or histidine residues in a peptide or protein during processing steps such as refolding or chromatographic procedures by including the compound according to formula I.
6. Method according to claim 4 or 5 in which the peptide is IGF-I.
7. Composition comprising peptides and proteins and a compound
10 according to formula (I) as antioxidant.
8. Composition according to claim 7 in which the peptide is a growth factor and preferably IGF-I.
- 15 9. Composition according to claim 7 or 8 comprising IGF-I and Ethionine, Butionine, 2-(methylthio)-ethanol, S-methyl-L cysteine and/or Methionine as antioxidant.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/01302

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/30, A61K 47/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REG, CAPLUS, WPI, MEDLINE, EMBASE, DPCI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9215614 A1 (CHIRON OPHTHALMICS, INC.), 17 Sept 1992 (17.09.92) --	1-9
A	US 5358708 A (SUMAN T. PATEL), 25 October 1994 (25.10.94) -- -----	1-9



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 January 1997

Date of mailing of the international search report

01-02-1997

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Gómez Lagerlöf

Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/10/96

International application No.

PCT/SE 96/01302

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9215614	17/09/92	AU-A- 1556492 US-A- 5272135	06/10/92 21/12/93
US-A- 5358708	25/10/94	NONE	